Nitric Oxide Enhances Angiogenesis via the Synthesis of Vascular Endothelial Growth Factor and cGMP After Stroke in the Rat

Ruilan Zhang, Lei Wang, Li Zhang, Jieli Chen, Zhenping Zhu, Zhenggang Zhang, Michael Chopp

Abstract—We investigated the effects of NO on angiogenesis and the synthesis of vascular endothelial growth factor (VEGF) in a model of focal embolic cerebral ischemia in the rat. Compared with control rats, systemic administration of an NO donor, *DETANONOate*, to rats 24 hours after stroke significantly enlarged vascular perimeters and increased the number of proliferated cerebral endothelial cells and the numbers of newly generated vessels in the ischemic boundary regions, as evaluated by 3-dimensional laser scanning confocal microscopy. Treatment with *DETANONOate* significantly increased VEGF levels in the ischemic boundary regions as measured by ELISA. A capillary-like tube formation assay was used to investigate whether *DETANONOate* increases angiogenesis in ischemic brain via activation of soluble guanylate cyclase. *DETANONOate*-induced capillary-like tube formation was completely inhibited by a soluble guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ). Blocking VEGF activity by a neutralized antibody against VEGF receptor 2 significantly attenuated *DETANONOate*-induced capillary-like tube formation. Moreover, systemic administration of a phosphodiesterase type 5 inhibitor (Sildenafil) to rats 24 hours after stroke significantly increased angiogenesis in the ischemic boundary regions. Sildenafil and an analog of cyclic guanosine monophosphate (cGMP) also induced capillary-like tube formation. These findings suggest that exogenous NO enhances angiogenesis in ischemic brain, which is mediated by the NO/cGMP pathway. Furthermore, our data suggest that NO, in part via VEGF, may enhance angiogenesis in ischemic brain. (*Circ Res.* 2003;92:308-313.)

Key Words: nitric oxide ■ phosphodiesterase type 5 inhibitor ■ vascular endothelial growth factor ■ angiogenesis ■ cerebral ischemia

Treatment of stroke with nitric oxide (NO) donors reduce functional neurological deficits.[1] NO is a pleiotropic molecule that affects many physiological and pathophysiological functions.[2] Animals treated with NO donors evoke cell proliferation in neurogenic regions of the brain, such as the subventricular zone and the dentate gyrus.[1] However, the mechanisms underlying the improvement of neurological function after treatment require clarification.

A potential therapeutic target for NO treatment of stroke is angiogenesis.[3] Administration of proangiogenic agents, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), to animals with stroke significantly reduce neurological dysfunction.[4,5] Incubation of human vascular smooth muscle cells with NO donors increases VEGF synthesis and the NO synthase (NOS) antagonist N^{ω}-nitro-L-arginine methyl ester (L-NAME) reduces VEGF generation.[6,7] Endothelial NO synthase (eNOS)–deficient mice exhibit significant impairment of angiogenesis in the ischemic limb, indicating that NO modulates angiogenesis in ischemic tissue.[8] Thus, there appears to be a coupling between NO, VEGF, and angiogenesis. However, there have been no studies on the effects of NO donors on VEGF and angiogenesis after stroke. Accordingly, we tested the hypotheses that NO increases VEGF and enhances angiogenesis via a cyclic guanosine monophosphate pathway (cGMP) in a model of focal embolic cerebral ischemia in the rat.

Materials and Methods

All experimental procedures were approved by the Care of Experimental Animals Committee of Henry Ford Hospital.

Animal Model

Male Wistar rats (Charles River, Portage, Mich) weighing 320 to 380 g were used. The middle cerebral artery (MCA) was occluded by placement of an embolus at the origin of the MCA.[9]

Experimental Protocol

(1) To examine whether exogenous NO affects neovascularization in ischemic animals, we administered (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-aminoethyl) amino]diazen-1-ium-1,2-diolate (*DETANONOate*), an NO donor with a half-life of 57 hours under physiological

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conditions,[10] to ischemic rats. *DETANONOate* (0.4 mg/kg) was intravenously administered to rats (n=8) 24 hours after stroke and daily (ip) for an additional 6 consecutive days.[1] Ischemic rats (n=8) treated with the same volume of decayed *DETANONOate* were used as a control group. All rats were sacrificed 14 days after stroke. (2) To examine the effect of exogenous NO on brain levels of VEGF, *DETANONOate* (0.4 mg/kg) or saline was administered to ischemic rats (n=3 for each group) with the identical paradigm described in Protocol 1. These rats were euthanized 7 days after stroke. (3) To examine whether increases in cGMP promote angiogenesis in ischemic brain, a phosphodiesterase type 5 (PDE5) inhibitor that increases cGMP, Sildenafil dissolved in 3 mL of tap water (2 mg/kg), was fed to ischemic rats (n=8) at 24 hours after stroke and daily for an additional 6 days.[11] Rats were euthanized 14 days after stroke.

Bromodeoxyuridine Labeling

Bromodeoxyuridine (BrdU, Sigma Chemical), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used for mitotic labeling. BrdU (50 mg/kg) was injected (ip) daily for 13 consecutive days into ischemic rats starting 1 day after MCA occlusion.

Three-Dimensional Image Acquisition and Analysis

To examine neovascularization in ischemic brain, fluorescein isothiocyanate (FITC) dextran (2×10^6 molecular weight, Sigma; 0.1 mL of 50 mg/mL) was administered intravenously to the ischemic rats subjected to 14 days of MCAo. The brains were rapidly removed from the severed heads and placed in 4% of paraformaldehyde at 4°C for 48 hours. Coronal sections (100 μm) were cut on a vibratome. The vibratome sections were analyzed with a Bio-Rad MRC 1024 (argon and krypton) laser-scanning confocal imaging system mounted onto a Zeiss microscope (Bio-Rad), as previously described.[12] Seven 100-μm thick vibratome coronal sections at 2-mm intervals from bregma 5.2 mm to bregma −8.8 mm from each animal injected with FITC-dextran were selected. Eight brain regions in the ipsilateral and contralateral hemispheres were selected within a reference coronal section (interaural 8.8 mm, bregma 0.8 mm). These regions were scanned in 512×512 pixel (276×276 μm^2) format in the *x-y* direction using a 4× frame-scan average and 25 optical sections along the *z*-axis with a 1-μm step-size were acquired under a 40× objective. Vascular branch points, segment lengths, and diameters were measured in 3 dimensions using software developed in our laboratory.[12] Image acquisition and analysis were performed blindly.

Immunohistochemistry and Quantification

For BrdU immunostaining, DNA was first denatured by incubating brain sections (6 μm) in 50% formamide 2X SSC at 65°C for 2 hours and then in 2N HCl at 37°C for 30 minutes.[9] Sections were then rinsed with tris buffer and treated with 1% of H_2O_2 to block endogenous peroxidase. Sections were incubated with a mouse monoclonal antibody (mAb) against BrdU (1:1000, Boehringer Mannheim) overnight and incubated with biotinylated secondary antibody (1:200, Vector) for 1 hour.

To quantify BrdU immunoreactive endothelial cells, numbers of endothelial cells and numbers of BrdU immunoreactive endothelial cells in 10 enlarged vessels adjacent to the ischemic lesion were counted from each rat. Numbers of endothelial cells and BrdU immunoreactive endothelial cells in the ten vessels of the contralateral homologous area were also counted. Data are presented as percentage of BrdU immunoreactive endothelial cells to total endothelial cells in 10 enlarged vessels from each rat.

Vascular perimeters were measured on coronal sections immunostained with an anti-von Willebrand factor antibody as previously described.[13]

ELISA for VEGF

The ischemic boundary regions and homologous tissue in the contralateral hemisphere were dissected. The tissue was homogenized and centrifuged at 10 000*g* for 20 minutes at 4°C and the supernatant was collected. ELISA for VEGF in the supernatants was performed using a commercially available kit specific for rat VEGF (R&D Systems) according to the manufacturer's instruction.

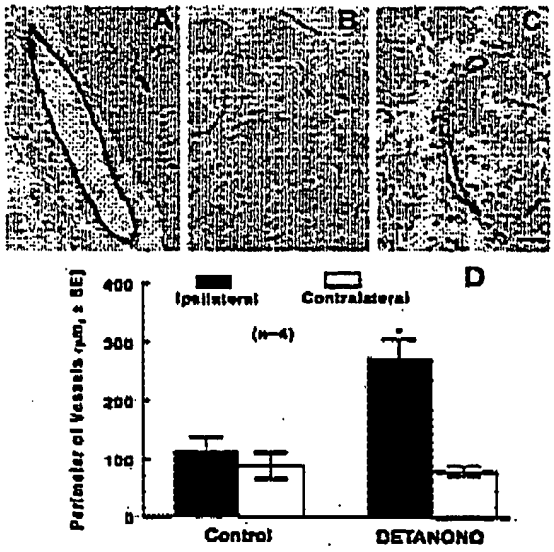

Figure 1. Cerebral vascular perimeters. Treatment with *DETANONOate* enlarged cerebral vessels in the ischemic boundary (A), but not vessels in the homologous area of the contralateral hemisphere (B) from a representative rat. C, Enlarged vessel in the ischemic boundary from a representative rat treated with decayed *DETANONOate*. Quantitative data (D) shows that treatment of stroke with *DETANONOate* significantly increased vascular perimeters compared with the ipsilateral vascular perimeters in the control rats. *$P<0.01$ vs ipsilateral. Bar in C=50 μm.

Capillary-Like Tube Formation Assay

An in vitro angiogenesis assay was performed.[14] Briefly, 0.8 mL of growth factor–reduced Matrigel (Becton Dickinson) was added to prechilled 35-mm culture dishes and allowed to polymerize at 37°C for 2 to 5 hours. Mouse brain–derived endothelial cells (2×10^4 cells)[15] were incubated for 3 hours in Dulbecco's modified Eagle's medium (DMEM) containing *DETANONOate*, Sildenafil, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), 8-Br-cGMP, or a rat anti-mouse neutralizing antibody to VEGF receptor 2 (VEGFR2, DC101, ImClone System). For quantitative measurements of capillary tube formation, 3 random areas of Matrigel dishes were imaged and the length of continuous cords of 3 or more cells was measured.[16]

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was used. The data were presented as mean±SE. A value of $P<0.05$ was taken as significant.

Results

Effects of *DETANONOate* and Sildenafil on Angiogenesis In Vivo

To examine whether exogenous NO enhances angiogenesis in ischemic brain, we administered *DETANONOate* to rats 24 hours after stroke for 7 days. Treatment with *DETANONOate* significantly ($P<0.01$) enlarged vascular perimeters (Figures 1A and 1D) around the ischemic lesion but did not enlarge

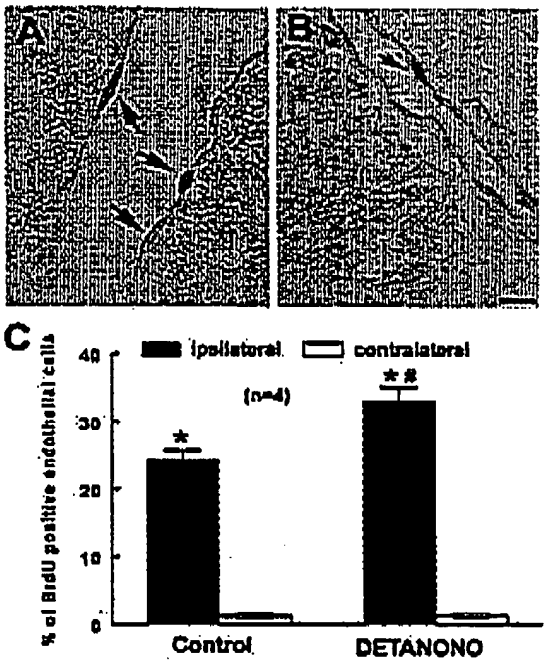

Figure 2. Proliferated cerebral endothelial cells. A, Several BrdU immunoreactive endothelial cells (arrows) in an enlarged thin-wall vessel of a representative rat treated with *DETANONOate*. B, BrdU immunoreactive endothelial cell (arrow) in an enlarged vessel of a representative rat from the control group. Although ischemia induced proliferation of endothelial cells (C, control), treatment with *DETANONOate* significantly increased the numbers of proliferated endothelial cells (C, DETANONO). *P<0.01 vs the contralateral hemisphere; #P<0.05 vs the ipsilateral hemisphere in the control group. Bar in B=10 μm.

vessels in the contralateral hemisphere (Figures 1B and 1D) compared with the ipsilateral vessels in the control rats (Figures 1C and 1D). Endothelial cells in enlarged thin-walled vessels exhibited BrdU immunoreactivity (Figures 2A and 2B) and quantitative analysis revealed that the numbers of proliferated endothelial cells significantly (P<0.05) increased in rats treated with *DETANONOate* (Figure 2C). To further examine angiogenesis, 3-dimensional analysis was performed using software developed in our laboratory, which measures numbers of segments, segment lengths, and diameters of vessels.[12] Treatment with *DETANONOate* significantly (P<0.05) increased the numbers of capillary segments in the boundary regions of ischemia (Figure 3A and Table) compared with the numbers in ischemic rats treated with same volume of decayed *DETANONOate* (Figure 3B and Table). The capillary segments in the *DETANONOate*-treated groups exhibited significantly smaller diameters (Figure 3A and Table) and shorter segment lengths (Figure 3A and Table), suggesting that these are newly generated vessels. A significant increase of angiogenesis was also detected in rats treated with Sildenafil (Table).

Effects of *DETANONOate* and Sildenafil on Brain Levels of VEGF

To examine whether administration of *DETANONOate* increases brain levels of VEGF, ELISA for endogenous rat VEGF was performed. ELISA measurements revealed that treatment with *DETANONOate* significantly (P<0.05) increased VEGF levels in the ischemic boundary regions from 13.4±1.5 pg/mL in the control group (n=3) to 28.9±1.0 pg/mL in the *DETANONOate*-treated group (n=3). Because NO increases cGMP, induction of VEGF by *DETANONOate* could occur via the cGMP pathway. PDE5 is highly specific for hydrolysis of cGMP. We, therefore, measured brain levels of VEGF in rats treated with the PDE5 inhibitor, Sildenafil. Treatment with Sildenafil significantly (P<0.05) increased VEGF levels (34.4±2.9 pg/mL versus 13.4±1.5 pg/mL in the control, n=3 per group) in the ischemic boundary.

Effects of Soluble Guanylate Cyclase Inhibitor and Neutralization of VEGFR2 on *DETANONOate*-Induced Capillary-Like Tube Formation

To support the hypothesis that *DETANONOate* increases angiogenesis in ischemic brain via the activation of soluble guanylate cyclase, we further analyzed the effects of *DETANONOate* on angiogenesis using a capillary-like tube formation assay. A significant increase in capillary-like tube formation was detected when mouse brain–derived endothelial cells[13] were incubated with *DETANONOate* (0.2 μmol/L;

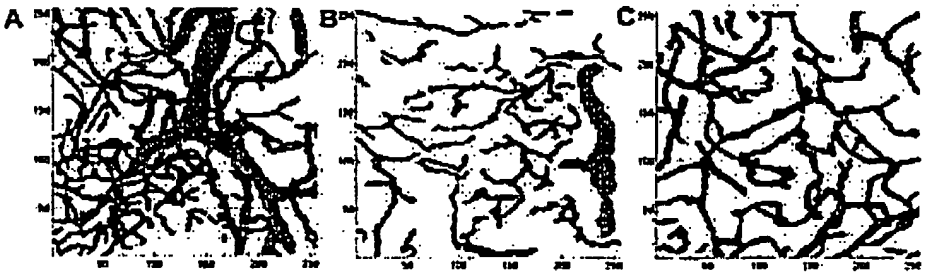

Figure 3. *DETANONOate* induces angiogenesis, as analyzed with 3-dimensional images. Computer-generated images were originally derived from images obtained with 3-dimensional laser scanning confocal microscopy. Treatment with *DETANONOate* increased the numbers of newly generated vessels (A), compared with the numbers of new vessels in rats in the control group (B). However, *DETANONOate* did not alter vascular morphology in the contralateral hemisphere (C). Green and red colors in the images represent vascular diameters larger and smaller than 7.5 μm, respectively. Image size is 276×276×25 μm^3, and the unit in the images is μm.

Three-Dimensional Quantitative Measurements of Vascular Morphology

	MCAo (n=4)		MCAo+DETANONO (n=4)		MCAo+Sildenafil (n=4)	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
No. of segments	38±6.3*	23±2.3	80±2.4*#	26±1.6	59±4.9*#	28±2.9
Diameter, μm	3.8±0.02*	4.5±0.04	3.4±0.02*#	4.2±0.03	3.7±0.01*#	4.3±0.02
Length, μm	26.5±0.66*	34.5±1.58	24.9±0.51*#	38.1±1.05	19.9±0.02*#	34.7±1.54

*P<0.05 vs the contralateral hemisphere; #P<0.05 vs the ipsilateral hemisphere of MCAo group.

Figures 4B and 4E) compared with the endothelial cells incubated with DMEM only (Figures 4A and 4E). However, *DETANONOate*-induced capillary-like tube formation was completely inhibited when the endothelial cells were incubated with *DETANONOate* in the presence of ODQ, a potent inhibitor of soluble guanylate cyclase[17] (Figures 4C and 4E), indicating that the NO/cGMP signalling pathway is involved in mediating the effects of *DETANONOate* on angiogenesis. To examine whether *DETANONOate* also enhances angiogenesis via increases in VEGF, the endothelial cells were incubated for 3 hours in the presence of *DETANONOate* (0.2 μmol/L) and a rat anti-mouse neutralizing antibody to VEGFR2 (DC101, 10 μg/mL). The biological activity of this antibody against VEGFR2 in the mouse has been demonstrated.[18] Treatment of endothelial cells with the antibody against VEGFR2 significantly ($P<0.05$) reduced *DETANONOate*-induced capillary-like tube formation (Figures 4D and 4E), suggesting that VEGF is involved in *DETANONOate*-induced angiogenesis.

Effects of Sildenafil on Capillary-Like Tube Formation

Incubation of the endothelial cells with Sildenafil (100 to 500 nmol/L) produced concentration-dependent capillary-like tube formation (Figure 5). 8-BrcGMP (1 mmol/L), a stable analog of cGMP, also significantly ($P<0.05$) increased capillary-like tube formation (Figure 5). ODQ (10 μmol/L) significantly inhibited Sildenafil-induced capillary-like tube formation (Figure 5), indicating that angiogenesis by Sildenafil is dependent on basal activity of sGC in the endothelial cells. ODQ did not significantly inhibit 8-BrcGMP–induced capillary-like tube formation (Figure 5), confirming that this effect is independent of soluble guanylate cyclase activation.

Discussion

The major findings of the present study are that (1) administration of *DETANONOate* or Sildenafil 24 hours after stroke increases synthesis of VEGF and enhances angiogenesis in ischemic brain; (2) ODQ, an inhibitor of soluble guanylate cyclase, completely inhibits *DETANONOate*-induced capillary-like tube formation; (3) Sildenafil, an inhibitor of PDE5, induces capillary-like tube formation; and (4) blocking of VEGF activity by a neutralized antibody against VEGFR2 attenuates *DETANONOate*-induced capillary-like tube formation; Together, these data indicate that exogenous NO enhances angiogenesis in ischemic brain via the NO/cGMP-dependent pathway and an inhibitor of PDE5 (Sildenafil) augments angiogenesis. Our data also suggest a coupling of NO, VEGF, and angiogenesis.

NO plays an important role in angiogenesis.[3] However, there have been no studies on the effect of NO on angiogenesis in ischemic brain. Mice lacking eNOS exhibit severe impairment of spontaneous angiogenesis in response to limb ischemia, and administration of L-arginine accelerates angiogenesis.[3] In the present study, administration of *DETANONOate* significantly increased the numbers of enlarged vessels and proliferated endothelial cells in the ischemic

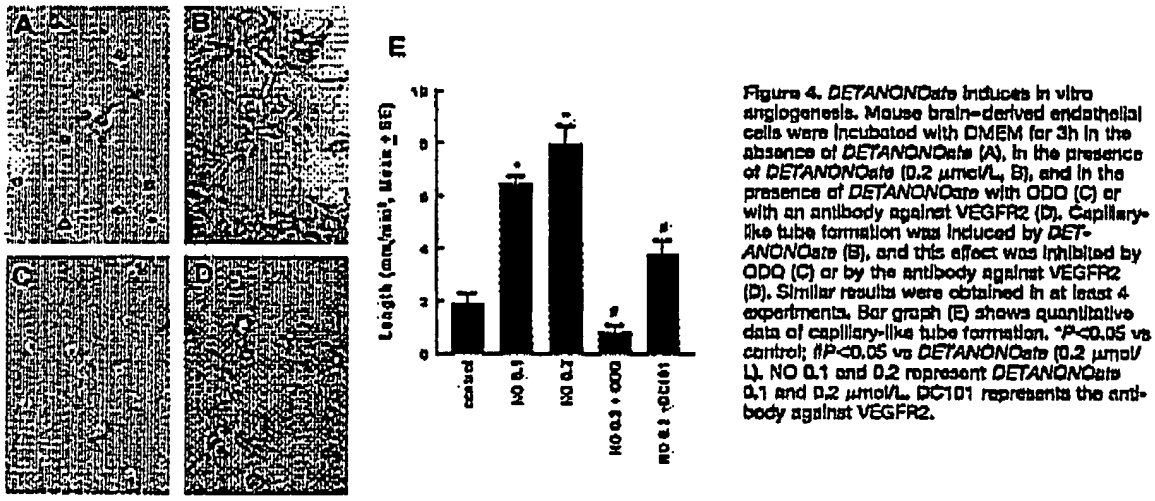

Figure 4. *DETANONOate* induces in vitro angiogenesis. Mouse brain–derived endothelial cells were incubated with DMEM for 3h in the absence of *DETANONOate* (A), in the presence of *DETANONOate* (0.2 μmol/L, B), and in the presence of *DETANONOate* with ODQ (C) or with an antibody against VEGFR2 (D). Capillary-like tube formation was induced by *DETANONOate* (B), and this effect was inhibited by ODQ (C) or by the antibody against VEGFR2 (D). Similar results were obtained in at least 4 experiments. Bar graph (E) shows quantitative data of capillary-like tube formation. *P<0.05 vs control; #P<0.05 vs *DETANONOate* (0.2 μmol/L). NO 0.1 and 0.2 represent *DETANONOate* 0.1 and 0.2 μmol/L. DC101 represents the antibody against VEGFR2.

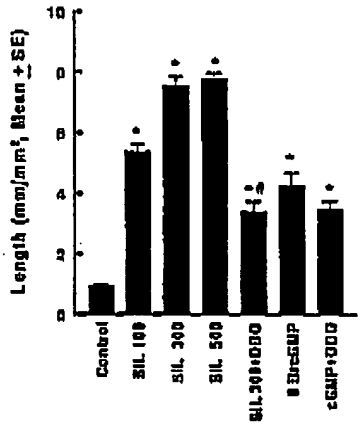

Figure 5. Bar graph shows quantitative data of Sildenafil-induced capillary-like tube formation. Sildenafil (100 to 500 nmol/L) and 8-BrcGMP induced capillary-like tube formation, and ODQ significantly inhibited Sildenafil (300 nmol/L)-induced capillary-like tube formation but did not attenuate 8-BrcGMP-induced capillary-like tube formation. *$P<0.05$ vs control; #$P<0.05$ vs Sildenafil 300 nmol/L. Sil indicates Sildenafil.

boundary regions, which is consistent with data that NO induces vessel dilation and endothelial cell proliferation.[19,20]

NO activates soluble guanylate cyclase, thereby producing an increase of cGMP in target cells.[21] PDE5 enzyme is highly specific for hydrolysis of cGMP, and Sildenafil citrate is a potent inhibitor of PDE5, which causes intracellular accumulation of cGMP.[22] In the present study, we show that *DETANONOate*-induced capillary-like tube formation was completely inhibited by ODQ, a selective inhibitor of soluble guanylate cyclase, suggesting that *DETANONOate* enhances brain angiogenesis via activation of soluble guanylate cyclase. Our results are in agreement with previous reports that NO activates soluble guanylate cyclase in angiogenesis.[23] To obtain further evidence that increases in cGMP contribute to NO-enhanced angiogenesis in ischemic brain, we administered the PDE5 inhibitor (Sildenafil) to rats 24 hours after stroke. Our data show that treatment with Sildenafil enhances angiogenesis in the boundary regions of ischemia. Moreover, Sildenafil and 8-BrcGMP (an analog of cGMP) induce capillary-like tube formation in a culture of brain-derived endothelial cells. ODQ significantly inhibits Sildenafil- but not 8-BrcGMP–induced capillary-like tube formation, indicating this response is dependent on basal activity of sGC. Therefore, our data support the conclusion that the NO/cGMP pathway mediates *DETANONOate*-induced angiogenesis in ischemic brain.

VEGF mediates angiogenesis[24] and NO and VEGF may interact to promote angiogenesis.[6,7] A high concentration of NO donor downregulates VEGF expression in endothelial cells.[25] In contrast, recent studies show endogenous NO enhances VEGF synthesis.[6,7] The eNOS-deficient mice exhibit significant impairment of angiogenesis in the ischemic hindlimb and administration of VEGF to these mice does not increase impaired angiogenesis, indicating that NO is a downstream mediator for VEGF-induced angiogenesis.[6,26] Angiogenesis in response to VEGF depends on the tissue microenvironments.[26,27] Our data show that exogenous NO increased ischemic brain levels of VEGF and blocking VEGF activity attenuated *DETANONOate*-induced capillary-like tube formation, suggesting that NO induces VEGF synthesis in brain and VEGF at least in part mediates *DETANONOate*-induced angiogenesis. These findings are consistent with previous studies that NO derived from NO donors can increase the synthesis of VEGF.[6] In addition, the PDE5 inhibitor, Sildenafil, increases brain levels of VEGF in the ischemic brain, suggesting that cGMP likely contributes to NO-induced VEGF synthesis. This finding is inconsistent with a previous study that the cGMP is not involved in NO-induced upregulation of VEGF in cultured human articular chondrocytes.[28] The reason for this discrepancy may be attributed to cell-type difference, but remains enigmatic.

Angiogenesis is tightly regulated by two families of growth factors, the VEGF and angiopoietin families, as well as endothelial cell interaction with extracellular matrix.[24] Upregulation of VEGF and angiopoietin genes are correlated with brain angiogenesis after stroke.[12] Furthermore, stroke induces expression of VEGF receptors 1 and 2 in endothelial cells of cerebral vessels.[12] Administration of NO-donor could amplify endogenous VEGF in the astrocytes and endothelial cells and consequently increased VEGF enhances angiogenesis in ischemic brain via interaction with upregulated VEGF receptors in the endothelial cells, as we previously demonstrated that treatment with VEGF increases angiogenesis in experimental stroke.[5] Newly generated vessels function in ischemic brain, and they may contribute to functional recovery via improvement of long-term perfusion.[13,29] Therefore, the positive interaction between NO and VEGF suggests that combination treatment with an NO donor and VEGF may have synergistic effects on angiogenesis.

Acknowledgments

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